NOTE TO THE FILE
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Subject: Rhone-Poulenc Ag. Company's Bromoxynil-Tolerant Canola, Oxy-235 line

Keywords:
Canola, *Brassica napus*, Navigator Canola, Bromoxynil Tolerant, Ioxynil Tolerant, Herbicide- Tolerant, 3,5-di-bromo-4-hydroxybenzonitrile, 3,5-di-iodo-4-hydroxy-benzonitrile, Nitrilase, Oxynil Tolerant, *Klebsiella pneumoniae*.

Background:

Per Rhone-Poulenc Ag Co. request, a meeting was held on May 7, 1999, to discuss their approach for evaluating the safety of Navigator canola Oxy-235 line. In a submission dated May 10, 1999, Rhone-Poulenc provided summary information to support the safety assessment of their Navigator canola line (bromoxynil-tolerant canola).

Intended effect and food/feed use

Navigator canola contains the *oxy* gene (also known as the BXN® gene) isolated from the soil bacterium *Klebsiella pneumoniae* subsp. *ozaenae*. The intended effect of this genetic modification is to confer tolerance to the oxynil family of herbicides, which comprise bromoxynil (3,5-di-bromo-4-hydroxybenzonitrile) and ioxynil (3,5-di-iodo-4-hydroxybenzo-nitrile). These herbicides are registered for use mainly as broad leaf herbicides on many important crops including wheat, barely, oats, triticale, corn, popcorn, rice, sugar cane, grain sorghum, flax (linseed), onions and garlic. Oxynils are post emergence anti-dicot weed killers which act mainly by blocking photosynthesis in dicot weeds. The *oxy* gene encodes the enzyme nitrilase that hydrolyzes the Oxynil herbicides to non-phytotoxic compounds.

Molecular alterations and characterization

The *oxy* gene from *Klebsiella pneumoniae* subsp. *ozaenae* is transferred into the genome of Westar canola cells by *Agrobacterium*-mediated transformation. The introduced gene is transcribed and translated to produce a nitrilase enzyme. The plasmid from *Klebsiella* containing the *oxy* gene was isolated and used to transform *E. coli* to a bromoxynil utilization positive phenotype. The *oxy* gene was then subcloned on a 2.5 Kb *Pst*I restriction fragment.

The notifier used the non-phytopathogenic (disarmed) strain EHA101 of *Agrobacterium tumefaciens* containing the plant expression construct, RPA-BL-235 in vector pRPA-BL-235, in the transformation of Westar canola to produce the transformation event Oxy-235-2. The transformation vector is a double-border vector with the *oxy* gene (optimized for plant expression) located within the T-DNA. The vector contains well-characterized DNA segments required for selection and replication of the plasmid vector in bacteria and transfer of the T-DNA into plant cells. These elements were not transferred to Navigator canola. The expression of *oxy* gene is driven by a constitutive promoter (CaMV 35S) and is stabilized by the non-translated portion of RuBisCo SSU gene from maize. Expression of the *oxy* gene from the constitutive promoter occurs in all parts of the plants and at all stages of development. Transcription is terminated by the polyadenylation signal from the nopaline synthase gene from pTi 37. Transformed canola cells were selected for bromoxynil tolerance.

The Navigator canola (Westar Oxy-235) line is directly derived from an original R0 transformant, Oxy-235-2. To characterize the inserted T-DNA in terms of copy number, insert integrity (gene size), and absence of transfer downstream from the left border, Rhone-Poulenc performed Southern blot analyses of genomic DNA from leaf tissue of the transgenic Oxy-235 / R3 canola. The results presented in their safety assessment indicate that, in Oxy-235 line, there is one copy of the *oxy* gene (1.15 Kbp fragment) inserted at a single site. No sequences downstream of the left border were transferred to the plant genome. Rhone-Poulenc sequenced the plant DNA at the site of insertion to confirm the absence of plasmid sequences other than the T-DNA.

The R1 seeds of Oxy-235 canola showed Mendelian segregation of the bromoxynil tolerance trait (3:1 ratio of susceptible:tolerant plants). These results confirm the insertion of the *oxy* gene at a single locus. To assess the stability of the inserted DNA in the Oxy-235 line, Rhone-Poulenc studied the inheritance of the bromoxynil tolerance in progeny produced by self-pollination of the transgenic plant and in a backcross program involving introgression of the *oxy* gene into a variety of genetic backgrounds. Evidence of genetic stability in homozygous strains of Oxy-235 canola is provided by maintenance of the oxynil tolerance trait over successive generations. Rhone-Poulenc also performed Southern blot analysis on genomic DNA from Samouri-Oxy-235 (a transgenic winter elite line of canola) and Westar-Oxy-235/R3. The results indicated that the *oxy* gene is located in the same site in the genomes of the two lines.

Characterization of the *oxy*-encoded nitrilase

Rhone-Poulenc measured the level of expression of nitrilase in different tissues (leaf and seed) from non-transgenic Westar and Westar-Oxy-235/R3 canola by Western blotting. Data indicate that Westar-Oxy-235 canola contains about 1000 ng of nitrilase protein per mg of extractable protein in leaf tissue and about 2 ppm in the meal. The nitrilase protein could not be detected in oil at a detection level of 20 ppb.

Rhone Poulenc states that nitrilase exhibits optimum activity in sodium carbonate buffer at pH 9.2, over a broad range of temperatures. Tests with several nitrile compounds show that the

oxy encoded nitrilase is specific for oxynil herbicides.

Allergenic and toxic potential

In order to assess a potential toxicological or allergenic effect of the oxy nitrilase, Rhone-Poulenc conducted a search in databases for similarity with proteins known for their toxicity or allergenicity. The nitrilase amino acid sequence showed no significant homology to known toxins and allergens. Furthermore, nitrilase doesn't share characteristics common to allergens.

The notifier conducted digestive fate studies with the nitrilase, in simulated gastric and intestinal fluids and concluded that the enzyme is subject to protease degradation. Nitrilase is present at very low amounts in unprocessed seed, and in canola meal. The exposure of animals to nitrilase from Oxy-235 canola is expected to be insignificant and there is no evidence that nitrilase is toxic. To confirm the absence of toxic effect of nitrilase, Rhone-Poulenc conducted a 14-day sub-acute toxicity feeding study in mice with bacterial nitrilase. According to the notifier, no toxic effects were observed.

Glucosinolates and erucic acid are naturally occurring endogenous toxicants in canola and are considered to be undesirable, anti-nutritional compounds of concern for food and feed safety. Feed regulations specify that canola meal may contain a maximum of 30 µmoles of any mixture of 3-butenyl (BUT), 4-pentenyl (PEN), 2-hydroxy-3-butenyl (HOBUT) and 2-hyroxy-4-pentenyl (HOPEN) glucosinolates per gram of air-dry, oil-free solid (The Association of American Feed Control Officials, 1999). Rhone-Poulenc measured the levels of glucosinolate composition of seeds from: control Westar, Westar Oxy-235, Westar Oxy-PN (a pure-breeding oxynil-tolerant population), and the precommercial elite control Tanto and Tanto Oxy canola lines. The notifier concluded that the glucosinolate levels in the Oxy lines are equal or lower than those in control lines and that they are comparable to the values reported in the literature.

Canola by specification, has 2% or less of its fatty acids components as erucic acid (C22:1) in the oil (21 CFR 184.1555, 1992). Rhone-Poulenc presented the levels of the naturally-occurring erucic acid (as percent of total fatty acids) in oil from: control Westar, Westar Oxy-235, Westar Oxy-PN, and the elite control Tanto and Tanto Oxy canola lines. Rhone-Poulenc concluded that the genetic modification has not altered the levels of erucic acid in oil derived from the Oxy lines.

Nutritional assessment

The intent of the genetic modification made by Rhone-Poulenc was to produce bromoxynil-tolerant canola. Rhone-Poulenc did not anticipate any other effect from the introduction of the nitrilase gene into canola. Nonetheless, Rhone-Poulenc conducted extensive compositional analyses of oil and seeds from the non-transgenic and the Oxy-235 Westar canola lines to confirm that there were no unintended effects of the genetic modification.

Oil:

Canola seeds are used to produce refined canola oil for human consumption. Canola produces a high-quality oil that appears to have superior cooking characteristics. Canola oil offers an excellent mix of fatty acids; it is low in saturated fat and provides moderate amounts of polyunsaturated fatty acids. Therefore, Rhone-Poulenc analyzed the fatty acid composition of oil from control Westar, Westar Oxy-235, Westar Oxy-PN, and the elite control Tanto and Tanto Oxy lines. The values obtained are all within the range for quality standards of canola. Rhone-Poulenc reported that the genetic modification has not significantly altered the fatty acid composition of the genetically modified canola relative to its counterparts. The notifier also concluded that the molecular modification did not change the levels of sterols, tocopherols, and unsaponifiable matter in crude oil.

Meal:

The feed quality of canola meal is based on its high protein content and suitable amino acid composition and limited by its fiber content and antinutritional factors. Canola meal is used as a protein supplement in animal feed (poultry, swine, beef, and dairy cattle). Rhone-Poulenc concluded that the nutritional profile of seeds from control Westar and Westar Oxy-235 canola lines was comparable to the published canola data and that the genetic modification has not influenced the nutritional value of the canola. The measured nutrient profile included: dry matter, ashes, crude fiber, neutral detergent fiber (NDF), acid detergent fiber and lignin (ADF and ADL), nitrogen, crude protein, total fat, soluble sugars, amino acid content, as well as gross energy level in seeds and meal.

To demonstrate the absence of toxic effects from canola seeds, Rhone-Poulenc conducted a 28-day rat feeding study where 5 male and 5 female rats were fed canola meals from Westar Oxy-235 (treated or not treated with bromoxynil), Westar, or Tanto control lines. No toxicity was observed in animals receiving up to 10% canola meal in their food regardless of the source of the meal.

Conclusions

Rhone-Poulenc Ag Company has concluded that its bromoxynil-tolerant canola line Oxy-235 containing transformation event 235-2 is not materially different in terms of food safety and nutritional profile from canola varieties currently on the market. At this time, based on Rhone-Poulenc's description of its data and analysis, the agency considers Rhone-Poulenc's consultation on Oxy-235 canola line to be complete.

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